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The influence of various aminoglycoside preparations on bilirubin/albumin binding

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The clinical importance of the displacement of bilirubin from the binding to albumin in newborns by drugs was first recognized by SILVERMAN et al. [25] in 1954–55 for sulfisoxazol (Gantrisin®). Since then sulfonamides are not given to icteric newborns.

Gentamicin and other aminoglycosides are frequently used for severe systemic infections of newborn infants. Aminoglycosides bind to only a low proportion to plasma protein; therefore, a competitive displacement of bilirubin from albumin was considered a priory unlikely for these antibiotics. Nevertheless, some preliminary communications indicated that gentamicin in icteric newborns competes with bilirubin for albumin binding [6, 13, 19, 27]. Other investigators [16, 29, 30] were unable to demonstrate such effect in various experimental designs. ODELL et al. [20] noted in a 1975 commentary, that they were unable to reproduce their own results from the years 1972/73 when in part different gentamicin preparations had been tested. During the past years we have tested the tolerance of various drugs in young icteric Gunn rats [1, 15, 23, 26]. This report will summarize the results with various gentamicin preparations and several other aminoglycosides. For the experiments we used the drugs as they are marketed in ampules rather than the pure substances. Thus, the results include the effect of additives (solvents and preservatives) which will be designated as stabilizers (STAB).

Curriculum vitae

LEONORE BALLOWITZ *was born in 1923 in Lichtenberg. She obtained her medical degree from the University of Berlin in 1945. Since 1948 she is on the staff of the Kaiserin Auguste Victoria House, the Children's Hospital of the Free University of Berlin. Currently she is the director of the Department for Neonatology and Intensive Care. She qualified as Privatdozent in 1956 and her scientific work is primarly in the areas of blood group incompatibility, neonatal bilirubin metabolism, phototherapy and neonatal defense mechanisms against infections.*

The results of our investigation caused MERCK Company in early 1975 to remove the stabilizer from the 10 mg ampules of Refobacin®. We have tested primarily Refobacin® 10 mg ampules from the production of 1973/74 and those from 1975 in comparison, later on.

1 Methods

Homozygous Gunn rats, 3–5 days and one month old respectively, served as experimental animals. As genetically determined inborn error of metabolism they lack glucuronyl transferase. They cannot couple bilirubin to glucu-

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ronic acid and suffer from a life-long hyperbilirubinemia and they usually develop a mild to moderate kernicterus during the first two weeks of life. Its extent can be determined histochemically in the PURKINJE' cells of the cerebellum.

Tab. I tabulates the preparations tested and the substances contained in the stabilizers as communicated to us by the manufacturers. In addition to the commercial preparations we investigated the stabilizers of the 40 mg Refobacin® and the 10 and 40 mg Sulmycin® ampules alone as well as the separate ingredients of the stabilizers benzyl alcohol and di-Na-EDTA.

In a pilot study, we determined in heterozygous (non icteric) young Gunn rats, the LD 50, i. e. the acute toxicity of the several preparations according to the method of BEHRENS and KÄRBER. The results were calculated from the survival rate four days after the injection.

Experimental Series 1 contained 3–5 day old homozygous animals who received a single high dose (50–75% of the LD 50 for heterozygous animals) of the particular test preparation subcutaneously. Prior to the injection, and 30 minutes, 3 hours, 24 hours, and in some instances 48 hours after the injection, the serum bilirubin concentration was determined in blood from the tail (direct photometric method with American Optical Bilirubinometer).

In order not to stress the neonatal rats too much by frequent blood drawings the neonatal rats were tested usually only three times. In all animals the first sample was obtained before the injection, the second sample was obtained in some animals after thirty minutes and others after three hours and the third sample in most animals after 24 hours with several at 48 hours. The graphic illustrations equate the pre-injection value with 100 and de-

Tab. I.

Tested preparations	Contents per ampule	Additives in ampules according to manufacturers' information	per	ml
A. GENTAMICIN				
Refobacin® X 40 mg[1]	1 ml	Na-pyrosulfite	3.2	mg
		di-Na-EDTA	0.1	mg
		benzyl alcohol	10.0	mg
Refobacin® 10 mg (Prod. 1973/74)[1]	2 ml	di-Na-EDTA	0.1	mg
		NaCl	5.0	mg
		benzyl alcohol	10.0	mg
Refobacin® 10 mg (Prod. 1975)[1]	2 ml	none		
Refobacin® 5 mg L for intrathecal application[1]	1 ml	none		
Sulmycin® 40 mg[2]	1 ml	methylparaben[7]	1.80	mg
		propylparaben[8]	0.20	mg
Sulmycin® 10 mg[2]	1 ml	methylparaben	1.30	mg
		propylparaben	0.20	mg
Garamycin® (diagnostic reagent, pure substance)[3]		none		
B. Other AMINOGLYCOSIDES				
Kanamycin® 1 g[4]	3 ml	methylparaben	0.6	mg
		propylparaben	0.07	mg
Gernebcin® 40 mg (Tobramycin)[5]	1 ml	Na-disulfite	3.2	mg
		phenol	5.0	mg
		di-Na-EDTA	0.1	mg
Sisomicin 75 mg[6]	1.5 ml	Na-metabisulfite	3.0	mg
		di-Na-EDTA	0.1	mg
		methylparaben	0.8	mg
		propylparaben	0.1	mg
		NaCl	3.6	mg
Sisomicin 20 mg[6]	2 ml	Na-metabisulfite	1.484	mg
		di-Na-EDTA	0.1	mg
		methylparaben	1.3	mg
		propylparaben	0.2	mg
		NaCl	6.372	mg
Sisomicin 4 mg for intrathecal application[6]	1 ml	none		

Manufacturers: [1] MERCK; [2] BYK ESSEX; [3] SCHERING Corp.; [4] GRÜNENTHAL; [5] LILLY; [6] BAYER test preparation-trade name: Extramycin; [7] methylparaoxybenzoic acid; [8] propylparaoxybenzoic acid.

pict the subsequent values as percent thereof. The base line bilirubin ranges between 7 and 10 mg% (rarely up to 11 mg%). Standard deviations for the pups of one litter was usually less than 1 mg%.

As further parameters of the toxicity of the several drugs the mortality (i. e. 4 day survival) and the weight gain were determined.

Experimental Series 2 comprises 3 to 4 week old homozygous Gunn rats. They were treated with some of the drugs in the same dosage as animals of series 1. Serum bilirubin concentrations were determined at similar intervals and the curves were compared with those of the newborn rats.

Experimental Series 3 comprised 3–5 day old homozygous Gunn rats who received several low dose injections of various drugs at 24 hour intervals. Weight gain, mortality and histochemical evidence for damage to the PURKINJE' cells in the cerebellum were determined. Serial bilirubin determinations were not obtained.

The animals were killed 24 hours after the last injection by decapitation. The brains were removed, weighed and frozen in CO_2 at - 70° C. Frozen sections of 8–10 micron thickness were stained with the following methods: hematoxilin-eosin, lactate dehydrogenase (LDH), $NADH_2$-tetrazolium-reductase (DPN-Diaphorase, $NADH_2$-R) (Barka and Anderson 1965). Preparations incubated without substrate served as controls.

With the aid of the enzyme reaction, the cytoplasm stains as a fine granular blue reaction product while the nucleus remains unstained. Cells with a metabolism impaired by bilirubin do not stain [9]. The damage to PURKINJE' cells thus demonstrated served as an indication for the degree of the bilirubin encephalopathy. It must be taken noticable damage to their PURKINJE' cells between the 8th and 14th day of life even without drugs. Therefore, we have graded the PURKINJE' cell damage by establishing two groups. The first group comprises animals which have a loss of PURKINJE' cells (PCL) expected for Gunn rats of a given age. The other group comprises animals which demonstrated a further loss of cells. Heterozygous and homozygous Gunn rats of the same litter served as a control after receiving injections of normal saline.

2 Results

2.1 LD_{50} for 3–5 day old heterozygous Gunn rat

Tab. II lists the LD_{50} for heterozygous non-icteric young rats as determined by us. It confirms data from the literature that the tolerance for kanamycin is twice as high as for gentamicin but that sisomicin is tolerated only half as well. Tobramycin is somewhat better tolerated than gentamicin. It is remarkable that the LD_{50} differs not only between the various aminoglycosides but also within ampules of different pharmaceutical preparations of the same active ingredient. The difference with Sulmycin 10 and 40 mg ampules does not appear to be too marked in Tab. II. Nevertheless, only three of 18 animals which received 600, 650 and 700 mg/kg from the 10 mg ampules remained alive as opposed to 8 out of 13 receiving identical

Tab. II. Acute toxicity of various tested preparations for 3–5 day old heterozygous Gunn rats.

	LD_{50} [1]	Amount of stabilizer injected simultaneously	
Refobacin® 40 mg	650 mg/kg	0.016	ml/g
Refobacin® 10 mg (Prod. 1973/74)	375 mg/kg	0.075	ml/g
Refobacin® 10 mg (Prod. 1975)	625 mg/kg	–	
Refobacin® L 5 mg lyophilized	700 mg/kg	–	
STAB[2] Refobacin® 40 mg		0.07	ml/g
Benzyl alcohol 10 mg/ml		0.07	ml/g
Sulmycin® 40 mg	675 mg/kg	0.017	ml/g
Sulmycin® 10 mg	550 mg/kg	0.055	ml/g
STAB[2] Sulmycin 40 mg		> 0.06	ml/g
STAB[2] Sulmycin 10 mg		> 0.08	ml/g
Kanamycin®	1500 mg/kg	0.0045	ml/g
Gernebcin® (Tobramycin)	850 mg/kg	0.021	ml/g
Sisomicin 75 mg	300 mg/kg	0.006	ml/g
Sisomicin 20 mg	350 mg/kg	0.035	ml/g
Sisomicin 4 mg lyophilized	300 mg/kg	–	

[1] LD_{50} was determined by injecting subcutaneously various doses at 50 and 100 mg steps into 20–50 animals. The values are rounded off.

[2] = stabilizer without the antibiotic.

amounts from 40 mg ampules. For sisomicin the variation between the three tested preparations is minimal. There are no recognizable correlations between the added stabilizers.

Very noticable is the difference for Refobacin® preparations. Following the injection of 600, 650 and 700 mg/kg from Refobacin® L 5 mg ampules (lyophilized for intrathecal administration) 20 out of 30 animals remained alive; with the same dose from 40 mg ampules 19 of 33 survived and with the same dose from 10 mg ampules produced in 1975 6 of 21 survived. In contrast none of 20 animals survived the lower dose of 400 or 500 mg/kg of Refobacin® 10 mg ampules produced in 1973/74.

Tab. II lists for each preparation the amount of stabilizer corresponding to the LD_{50} injected per gram body weight of the rats. With stabilizers containing benzyl alcohol or paraoxybenzoic acid derivatives (Paraben) toxicity can be recognized from about 0.05 ml/g. Evidently, for Refobacin® 10 mg (made in 1973/74) not the antibiotic but the benzyl alcohol content in the stabilizer determines the LD_{50}.

2.2 Administration of single high doses to neonatal homozygous Gunn rats

The maximal dose of various gentamicin preparations and tobramycin in Series 1 for homozygous young rats was 400 mg/kg, of kanamycin 1000 mg/kg. Except for Refobacin® 10 mg (produced 1973/74) in each group at least 70% of the animals survived at least four days after the injection. This corresponds to the survival chances of untreated homozygous control animals of this age group.

On the other hand, only those animals receiving tobramycin or kanamycin had a mean weight gain of over 5 grams per day corresponding to the weight gain of control animals. With the various gentamicin preparations the mean weight gain varied between 3.9 and 4.9 grams. For Sulmycin® a trend for impaired weight gain because of the stabilizer may be recognized. With Refobacin® 10 mg (produced 1973/74) only one of 26 animals survived the single dose of 200 mg/kg (corresponding to 0.04 ml/g STAB Refo 10). The other animals died usually 24 to 48 hours after the injection. A single dose of 100 mg/kg (0.02 ml/g STAB) was survived by 12 of 14 animals. Analogous results were obtained after subcutaneous injection of benzyl alcohol (10 mg/ml) alone or of STAB Refo 40 without the antibiotic. Benzyl alcohol in a dose of 0.02 ml/g was survived by 6 of 8 animals but 0.04 ml/g was survived by none of 13 animals. Eleven of 19 animals survived 0.02 ml/g STAB Refo 40 and one of 13 animals survived 0.04 ml/g. Weight gain was decreased after the injection of benzyl alcohol solution as well as after STAB Refo 40 and Refobacin® 10 mg (produced 1973/74).

Sisomicin was injected in a maximal dose of 200 mg/kg into homozygous animals based on the LD_{50} of heterozygous young rats. There was no increased mortality. There was no significant difference between the various commercial preparations. The average weight gain was 5.22 ± 1.11 grams.

Figures 1 to 13 demonstrate the influence of the various aminoglycosides and the stabilizers of the commercial preparations upon the serum bilirubin concentrations of young homozygous Gunn rats. Only the means are depicted. These data were not analyzed statistically because of the variations in regard to sampling times.

After the observations referred to above about the increased occurrence of kernicterus in prematures treated with sulfonamides, JOHNSON et al. [3, 11] reported in 1959 similar observations in young homozygous Gunn rats. In the animal model the decrease of the serum bilirubin concentration occurring soon after the drug administration was clearly demonstrated. ODELL [18] correlated this occurrence with the displacement of protein-bound bilirubin from the albumin bond by specific anions including sulfonamides. Our results can be interpreted similarly.

The first example is the lyophilized preparation Refobacin® L 5 mg without stabilizer for intrathecal use (Fig. 1). The serum bilirubin concentration is hardly affected at all by the injection. The slight decrease after 30 minutes corresponds to that seen in control animals with similar injections of normal saline. Similarly, control animals usually show somewhat higher serum bilirubin concentrations 24 hours after injection. Because of the enzyme defect in homozygous animals the serum bilirubin concentration rises 0.5 to 0.7 mg% per day between the third and sixth day of life. 200 and

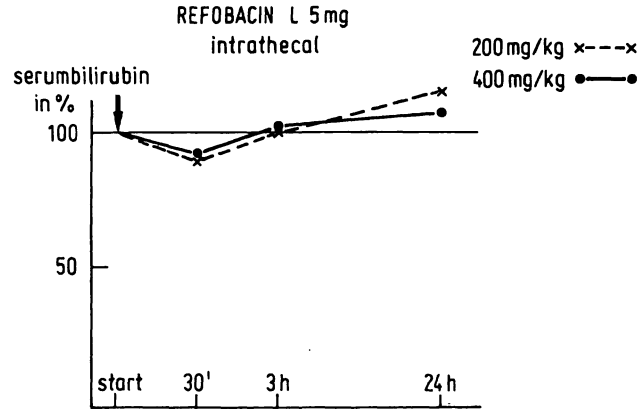
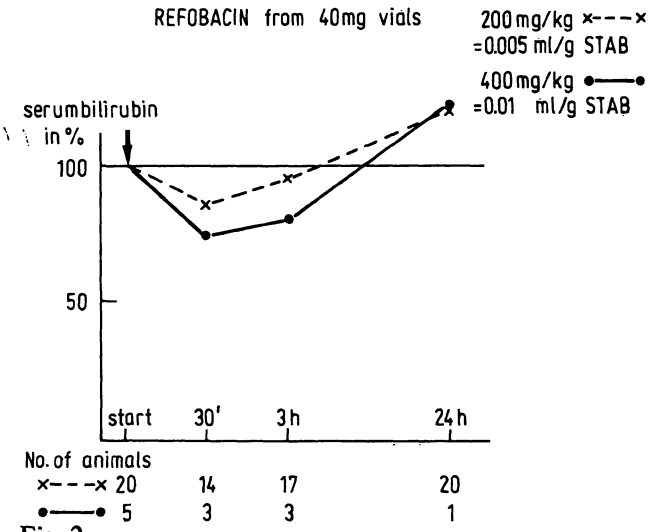


Fig. 1.



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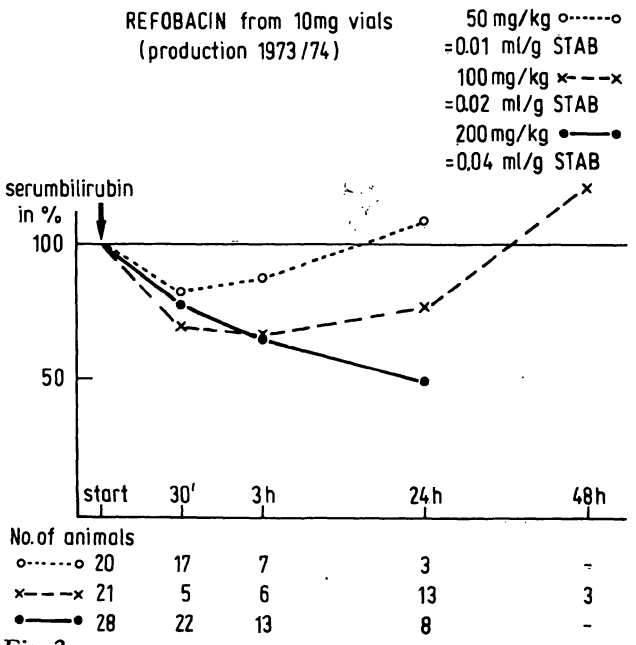

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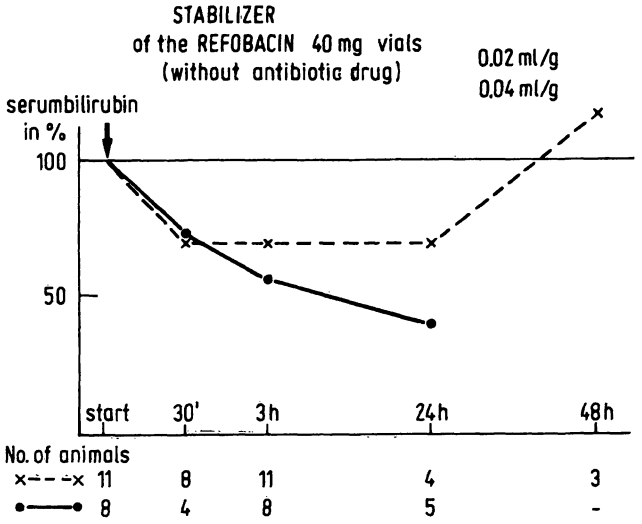

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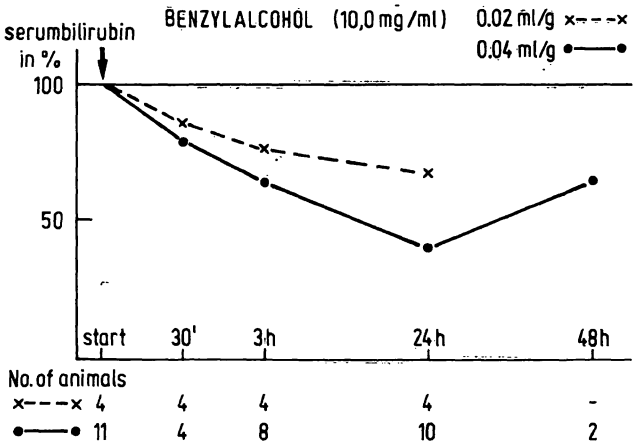

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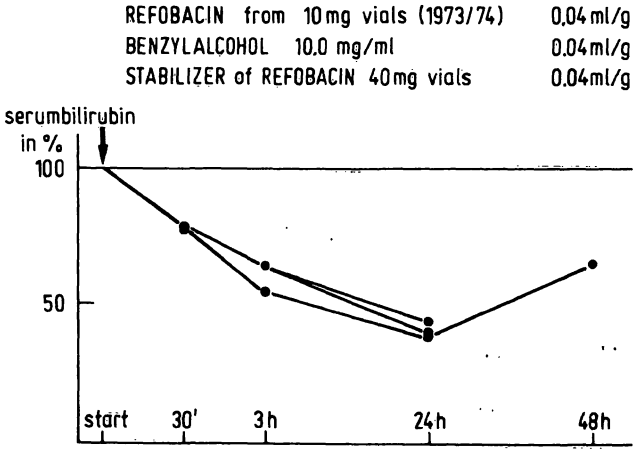
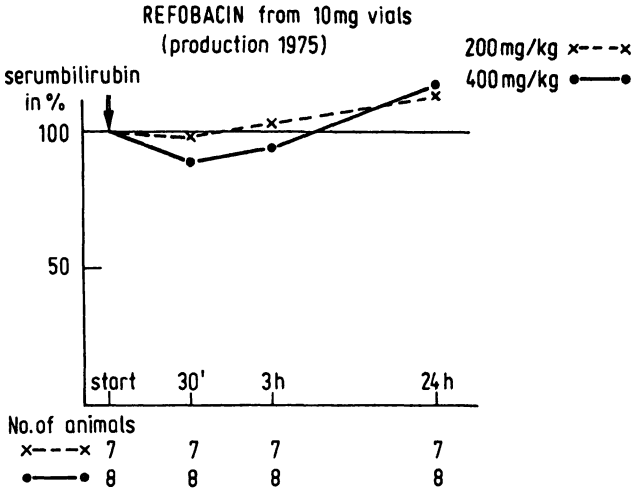


Fig. 6.

Fig. 1–6. Change in serum bilirubin concentration in 3–5 *day* old homozygous Gunn rats after one injection of antibiotics or stabilizer solutions.



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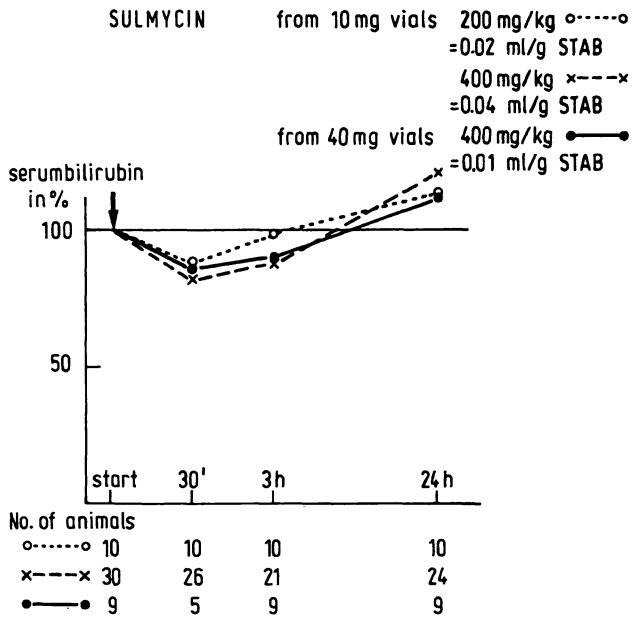

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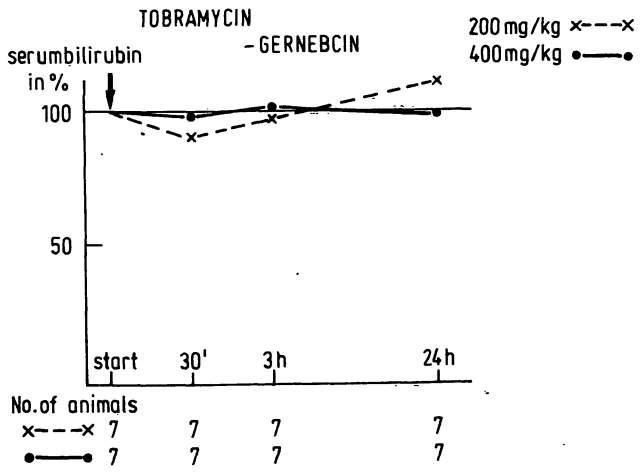

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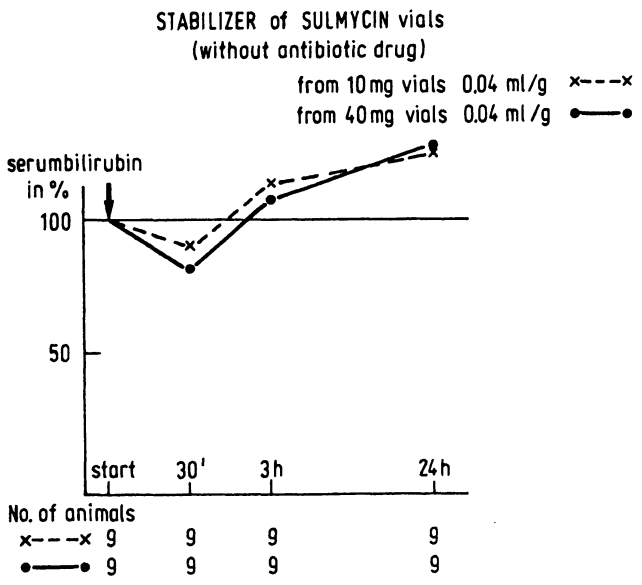

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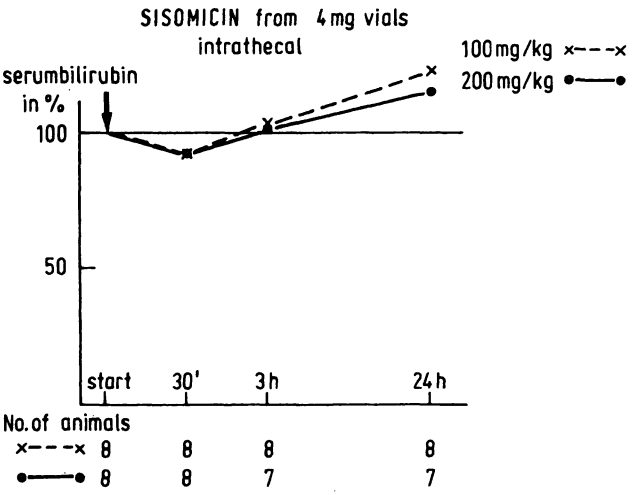

Fig. 11.

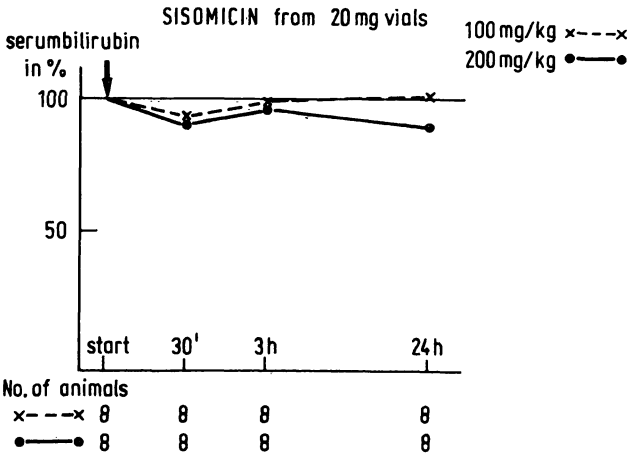

Fig. 12.

Fig. 7–12. Change in serum bilirubin concentration in 3–5 *day* old homozygous Gunn rats after one injection of antibiotics or stabilizer solutions.

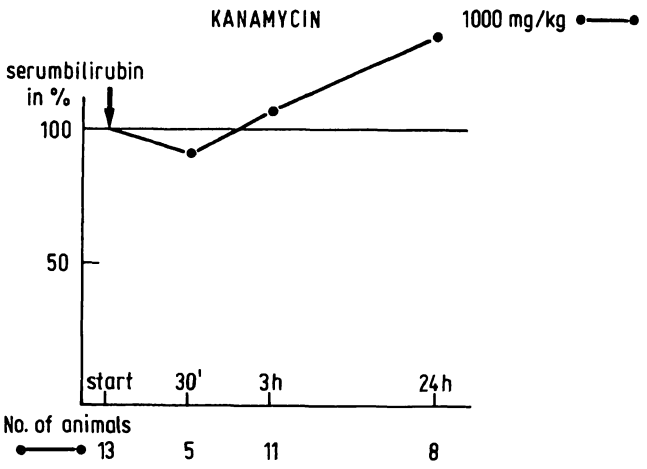

Fig. 13. Change in serum bilirubin concentration in 3–5 *day* old homozygous Gunn rats after one injection of Kanamycin.

400 mg/kg of gentamicin from Refobacin® 40 mg ampules (Fig. 2) caused a more pronounced drop in serum bilirubin, lasting longer than three hours. The decrease is even more pronounced with the higher dose.

As little as 50 mg/kg Refobacin® from 10 mg ampules manufactured in 1973/74 (Fig. 3) caused a drop in serum bilirubin similar in magnitude to that seen with 400 mg/kg from 40 mg ampules. This allows the assumption that the responsible factor is the associated injection of stabilizer in an amount of 0.01 ml/g. The serum bilirubin is lowered even more by 100 and 200 mg/kg from 10 mg ampules (1973/74 production), lasting for over 24 hours. Very similar curves are generated if STAB Refo 40 without antibiotic (Fig. 4) or only benzyl alcohol (Fig. 5) are adminstered. Fig. 6 demonstrates graphically the lines corresponding to 0.04 ml/g from the three preceeding illustrations. The bilirubin decrease coincides so well that the conclusion is justified that benzyl alcohol is the solely responsible agent. After injection of 200 and 400 mg/kg gentamicin from the new Refobacin® 10 mg ampules (1975 production) a similar influence on the serum bilirubin concentration is not seen (Fig. 7).

Curves obtained after gentamicin injections from Sulmycin® ampules (Fig. 8) as well as those after injection of stabilizers without the antibiotic (Fig. 10) indicate only a low bilirubin displacement. A clear dose related effect from the methyl and propyl-paraben in the stabilizers is not demonstratable.

Pure gentamicin (as the diagnostic reagent of SCHERING Corporation, Garamycin®) was tested in addition to the 200 and 400 mg/kg doses also in the amount of 5 mg/kg used in clinical practice. No indications for a displacement of bilirubin from plasma protein were found.

The curves for kanamycin (Fig. 13), tobramycin (Fig. 9), and sisomicin (Fig. 11 and 12) do not indicate bilirubin displacement. Di-Na-EDTA was tested as a single stabilizer substance; it does not influence the bilirubin concentration.

2.3 Administration of single high doses to 3–4 week old homozygous Gunn rats

In experimental series 2, three to four week old homozygous Gunn rats received 100 to 400 mg/kg gentamicin from Refobacin® L 5 mg ampules, 5 and 400 mg/kg from both Sulmycin® ampules as well as 5 and 100 to 400 mg/kg pure substance (Garamycin®). As in 3 to 5 day old animals there was no influence on the serum bilirubin concentration. Fig. 14 shows serum bilirubin curves of these older rats after injection of benzyl alcohol. The comparison with Fig. 5 (3–5 day old animals) demonstrates a steeper decrease in the first 30 minutes and a return to the base line values within 24 hours. Several older rats tolerated in contrast to the young rats 0.08 ml/g of the solution. A rather similar picture is obtained from Fig. 15. It demonstrates the bilirubin concentration of the older rats after gentamicin from Refobacin® 40 mg and Refobacin® 10 mg ampules (1973/74 production). In comparison with the young rats (Fig. 2 and 3) the primary bilirubin decrease is steeper, the return to the original values is quicker, and higher doses are tolerated.

The difference in the return of the bilirubin concentration indicates probably a different metabolic rate for benzyl alcohol in the two age groups because of the postnatal development of enzyme systems [31]. In order to explain the differences in the slope of the initial decrease, one could postulate for the younger animals either a decreased resorption or it could be assumed that the effect is not caused by the benzyl alcohol but by its metabolites.

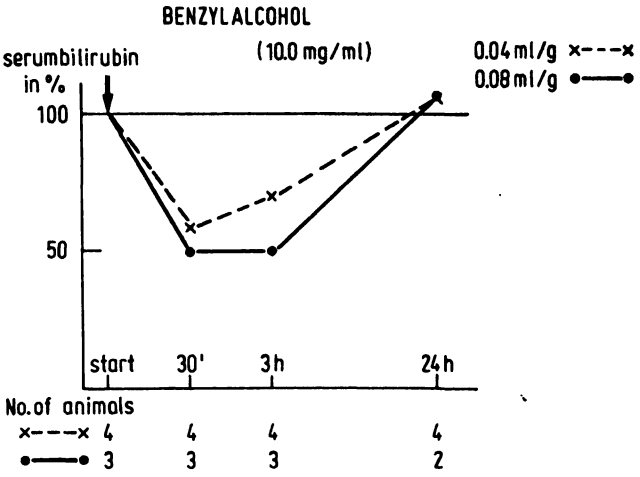

Fig. 14.

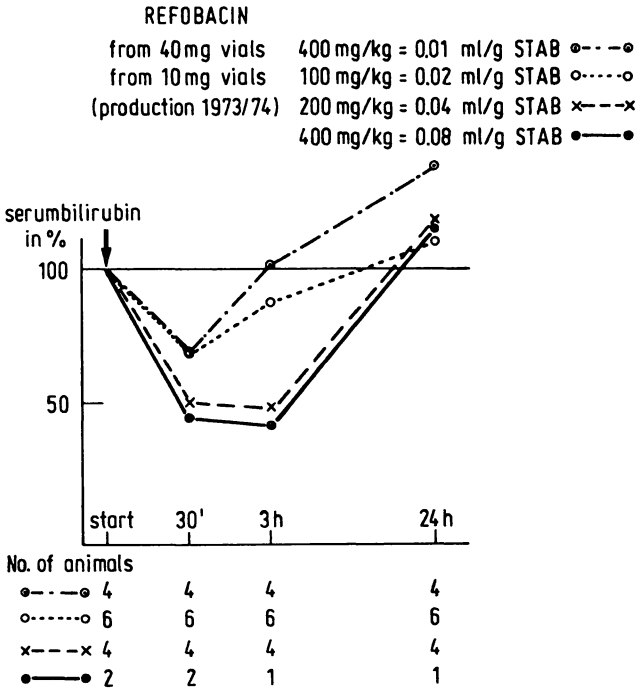

Fig. 15.

Fig. 14 and 15. Serum bilirubin concentration in 3–4 *week* old homozygous rats after injection of benzyl alcohol and Refobacin® from 40 mg and 10 mg ampules (1973/74 production).

2.4 Repeated injections in low doses to young hemozygous rats

Tab. III summarizes the survival of rats (3–5 days old at the beginning of the experiment) after one injection of gentamicin and after several injections repeated at 24 hour intervals (experimental series 3) from the three tested ampules for use in infants. The high mortality after one 200 mg/kg injection and five 100 mg/kg injections from Refobacin® 10 mg ampules (1973/74 production) is clearly evident. On the other hand, 21 of 24 animals survived who had received ten injections of 50 mg/kg of this preparation. The last column lists the mean weight gain for all animals who received ten injections if their base line weight was at least 9 grams. Here, too, Refobacin® 10 mg (1973/74 production) compares unfavorably with the other preparations.

Tab. IV summarizes findings in regard to the number of damaged PURKINJE' cells in the cerebellum of the animals. Ten to eleven days after the experiment when the rats were 13–16 days old the extent of the loss of PURKINJE' cells (PCL) in the vermis of the cerebellum has been estimated (Fig. 16–18). If animals who have had an increased PCL and those who died during the experimental period are added and compared to those with the amount of PCL expected for the age, the detrimental fact of Refobacin® 10 mg (1973/74 production) is quite evident. The dose of 100 mg/kg is not different in regard to the occurrence of kernicterus between 1 (not indicated in Tab. III) 2, and 5 doses; however, with 50 mg/kg a difference can be seen when 5 and 10 injections are compared. After ten 5 mg/kg Refobacin® 10 mg (1973/74 production) injections the adverse effect cannot be recognized. The extent of the damage to the ganglion cells after this low dose cannot safely be distinguished from that in animals who received normal saline solution or the other two gentamicin preparations in high doses.

3 Discussion

The various methods of determining displacement of bilirubin from albumin by drugs were compared by YEARY and DAVIS in 1974 [32]. Among the in vitro methods the separate sephadex-gel-filtration of proteinbound and unbound (so-called free) bilirubin proved particularly useful. Results obtained with this method corresponded well with the observed decrease in serum bilirubin concentration of icteric Gunn rats. The authors ascribe high potential for the testing of new drugs intended for use in the perinatal period to in vivo in-

Tab. III. Survival and weight gain of young homozygous Gunn rats after injection of gentamicin from ampules for use in infants.

	1 X 100 mg/kg	1 X 200 mg/kg	5 X 100 mg/kg	10 X 50 mg/kg	Weight gain in g after 10 injections in animals with initial weight over 9 g	Number of these animals
Refobacin® 10 mg (Prod. 1973/74)	12/14[1]	1/26	2/14	21/24	10.24 ± 2.98	8
Refobacin® 10 mg (Prod. 1975)		7/8	11/11	19/21	16.07 ± 1.3	12
Sulmycin® 10 mg		10/10	4/4	24/24	16.62 ± 0.52	9
				10 X 0.01 ml/g		
Normal saline				8/10	16.36 ± 0.86	8

[1] Enumerator: survivors/denominator: animals tested.

Tab. IV. Amount of PURKINJE' cell loss (PCL) in the cerebellum of homozygous young Gunn rats after injection of gentamicin from various ampules for use in infants.

Preparation	Dose	Number of treated animals	PCL expected for age	Increased PCL	Died
	100 mg/kg				
Refobacin® 10 mg Prod. 1973/74	2 X / 5 X	6/14:20	−/−:0	2/2:**4**	4/12:**16**
Refobacin® 10 mg Prod. 1975	2 X / 5 X	4/11:15	4/11:**15**	−/−:0	−/−:0
Sulmycin® 10 mg	2 X / 5 X	4/4:8	4/3:**7**	−/−:1	−/−:0
	50 mg/kg				
Refobacin® 10 mg Prod. 1973/74	5 X / 10 X	6/24:30	3/2:5	1/19:**20**	2/3:**5**
Refobacin® 10 mg Prod. 1975	5 X / 10 X	6/34:40	5/18:**23**	−/13:13	1/3:4
Sulmycin® 10 mg	5 X / 10 X	7/22:29	5/15:**20**	2/7:9	−/1:0
	5 mg/kg				
Refobacin® 10 mg Prod. 1973/74	10 X	22	**14**	7	1
	0.01 ml/g				
Controls: normal saline	10 X	23	**17**	4	2

vestigations with Gunn rats. The animal model allows a better determination of the relation between time, action, and dose of a drug than in in vitro tests and allows additional insight in the role of metabolites.

The risk of kernicterus from sulfonamides was identified first by clinicians through a careful controlled study in prematures. The animal model at that time helped to clarify the pathogenetic mechanism. The degradation of benzyl alcohol, benzoic acid and its derivatives is similar in man and rat. It leads to the formation of hippuric acid which is excreted in the urine. It may be expected that effects caused by these substances in rats may also occur in the human.

We began our drug studies in Gunn rats in 1973 primarily to verify the protective effect of phototherapy for kernicterus. We wanted to test wheth-

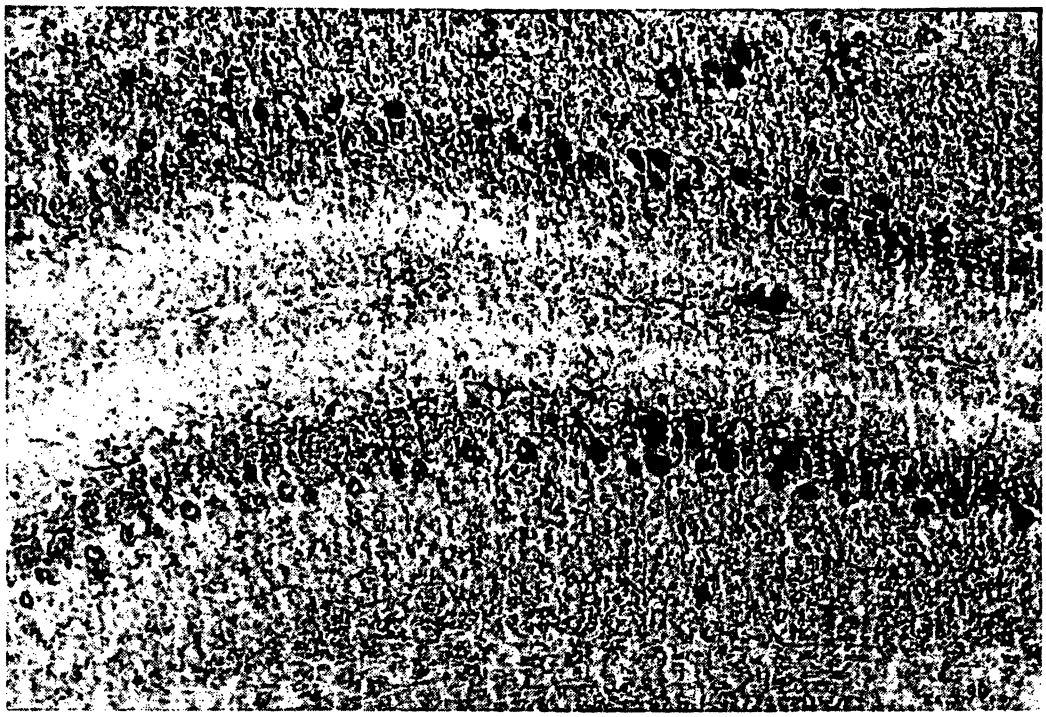

Fig. 16. Heterozygous Gunn rat, cerebellum, LDH, × 100. Normal number of PURKINJE' cells.

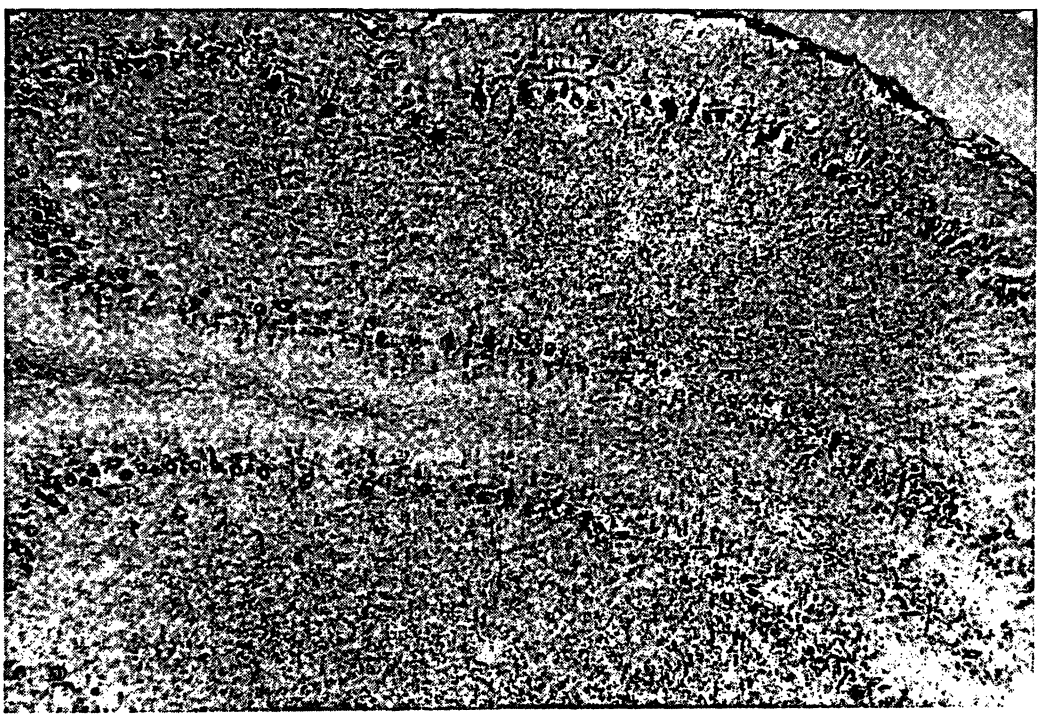

Fig. 17. Homozygous Gunn rat, cerebellum, LDH, × 60; 10 times 50 mg/kg Sulmycin® 10mg: minimal loss of PURKINJE' cells.

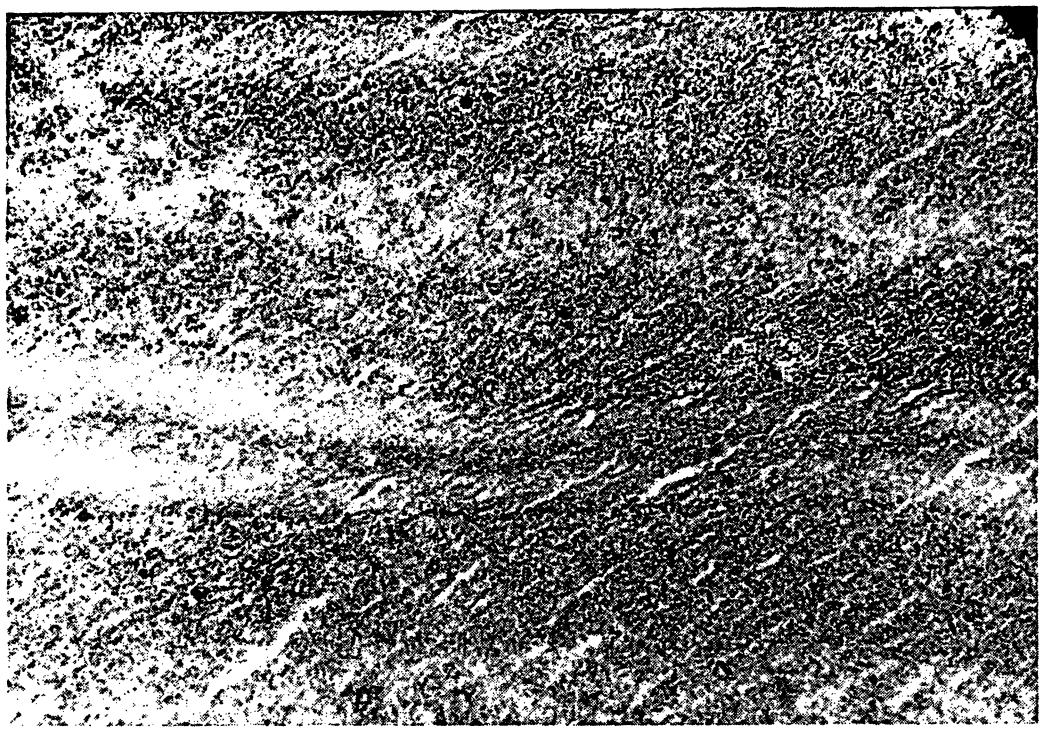

Fig. 18. Homozygous Gunn rat, cerebellum, LDH, × 60; 10 times 50 mg/kg Refobacin® 10 mg (1973/74 production): almost complete loss of PURKINJE' cells.

er the provocation of kernicterus by drugs may be prevented by simultaneous phototherapy and this question was soon answered in the affirmative. During the selection of substances which displace bilirubin from albumin and thus enhance the occurrence of kernicterus, we encountered gentamicin. When we began to use this antibiotic we were unaware of the differences in the preparations of the various commercial brands. In GERMANY additives do not have to be listed. Initially we used Refobacin® 10 mg and Refobacin® 40 mg ampules without particular preference according to the supply. Only after we recognized the marked difference in the acute toxicity and the different influence on the serum bilirubin concentration did we ask the manufacturer about the different additives in the ampules. From the work of BRATLID and LANGSLET [5] it was then known that ampules of diazepam of different origin do not displace bilirubin equally from the albumin bond. STERN and SCHIFF [27, 22] demonstrated that the bilirubin displacement from injectable Valium® was not caused by the diazepam, but by the benzoate in the stabilizer. They demonstrated a similar effect of the benzoate component in caffeine sodium benzoate. Thus, it appeared reasonable to investigate in particular the benzyl alcohol in the stabilizer of Refobacin® and the paraoxybenzoic acid ester in the stabilizers of Sulmicin® and the other tested aminoglycosides. The molecular weight of benzyl alcohol is 108, that of methylparaben 152, and of propylparaben 180. If it is assumed that the portion of the molecules responsible for protein binding is situated on the phenol ring the effect of parabene should be estimated as at least 1/3 less than that of benzyl alcohol. In a comparison of the ratio of the amounts of benzyl alcohol and benzoic acid ester (Tab. V) to that of the active ingredient it is evident that all ampules for use in infants contain more of them than the adult dosage form. This fact makes a general review of solutions for pediatric injections highly advisable. Furthermore, it is apparent that the amount of critical additives in Refobacin® 10 mg ampules (1973/74 production) is markedly higher than in all other preparations. It is by weight twice as high

Tab. V. Comparison of the ratio: Content of benzyl alcohol (BA) or paraben (PB) / Content of Antibiotic in various brands.

Preparation	mg benzyl alcohol (BA) or mg paraben (PB) per 10 mg antibiotic	Mass relation of the additives in ampules for adults	for infants
Refobacin® 40 mg	2.5 BA	1	8
Refobacin® 10 mg (Prod. 1973/74)	20.0 BA		
Sulmycin® 40 mg	0.50 PB	1	3
Sulmycin® 10 mg	1.5 PB		
Sisomicin 75 mg	0.18 PB	1	4
Sisomicin 20 mg	0.75 PB		

as the antibiotic! Thus, it is understandable that the addition of the stabilizer decreases the LD_{50} to half that of the pure substance (Tab. II). The LD_{50} of Sulmycin® 40 mg and Sulmycin® 10 mg ampules (Tab. II) can probably also be related to the stabilizer addition. In order to evaluate the noticably lesser difference in the LD_{50} of Refobacin® 40 mg, Refobacin® 10 mg (1975 production) and Refobacin® L 5 mg as well as sisomicin 75 mg and sisomicin 20 mg the findings must be corroborated with considerably larger numbers of experimental animals. This is an obligation of the manufacturers. Because of the considerable difference in the various ampules we think it imparative that the package inserts do not only list the LD_{50} of the drug as a pure substance, but also that of the specific preparation.

A further question is that of the relation between doses which damage Gunn rats and those in clinical use. Before the recognition of the bilirubin displacement, newborns received daily doses of 100 to 200 mg/kg of sulfisoxazol (Gantrisin®). A one time injection of these doses causes a decrease in bilirubin to about 50% of the base line value for longer than three hours in Gunn rats. An increased PCL can be demonstrated after only one injection of 100 mg/kg. After 400 mg/kg the bilirubin displacement persists for more than 24 hours. A decreased weight loss is recognizable after 1 X 600 mg/kg and an increased mortality after 1 X 800 mg/kg (i. e. 4 to 8 times the usual daily dose). Only few animals survive 1000 mg/kg.

NATHENSON and co-workers [17] studied the bilirubinlowering effect of sodium benzoate in Gunn rats in regard to the risk of the stabilizer in Valium® for newborns. They were able to demonstrate a decrease in serum bilirubin above that seen with NaCl injections only after several intraperitoneal injections of 35 mg/kg of sodium benzoate. This corresponds to about 3 mg/kg of Valium®, a high dose which is used in the treatment of neonatal seizures. However, the authors considered only the sodium benzoate in the Valium® stabilizer and disregarded the also present 10 mg benzyl alcohol. Only after 200 mg/kg sodium benzoate the bilirubin decrease resembled that seen with 200 mg/kg sulfisoxazol. In our laboratory we tested in early experiments (1, 26) the complete Valium stabilizer (without the active ingredient) in 3–5 day old rats. After subcutaneous injection of an amount containing 170 mg/kg sodium benzoate plus 50 mg/kg benzyl alcohol (in addition to propylen glycol and ethyl alcohol), the decrease in bilirubin corresponded to that after 200 mg/kg benzyl alcohol. NATHENSON et al. [17] concluded from their results that injectable Valium did not constitute a danger for newborns in regard to bilirubin displacement from albumin binding, especially considering the higher binding capacity of human albumin, as long as recommended doses were not grossly exceeded.

Newborns receive 3–5 mg/kg gentamicin per day and in sepsis up to 8 mg/kg. After treating young Gunn rats for ten days with daily doses of 5 mg/kg Refobacin® 10 mg (1973/74 production) we found no certain evidence of increased PCL. One injection of 50 mg/kg caused a bilirubin drop for longer than three hours to about 80 % of the original value. Animals receiving 50 mg/kg daily for ten days showed an increase PCL and the weight gain was reduced. After 100 mg/kg serum bilirubin remained decreased for over 24 hours to 65 % to

75 % of the base line value and the mortality was markedly increased (with a 10–20 times the clinical dose). Only one of 26 animals survived one injection of 200 mg/kg. It may be concluded that in newborns with a physiological level of albumin, and only moderate icterus the administration of Refobacin® from the 10 mg ampules (produced in 1973/74) alone in a moderate dose did not stress the albumin reserve binding capacity disconcertingly. However, with the co-occurrence of several important factors such as pronounced hypoalbuminemia, acidosis, decreased renal function, and the administration or endogenous accumulation of substances competing for protein binding, one cannot exclude competitive displacement of bilirubin and thus a neuronal damage by a superfluous additive.

In comparison to Refobacin® 10 mg/ampules (1973/74 production) the demonstrable effects of stabilizers in all other tested commercial preparations are minimal. The question whether they are indeed necessary for the preparation and stabilization of aminoglycoside solutions will not be discussed here. The fact that in Germany, additives in solutions or injections do not have to be declared even if their amount is higher than that of the active ingredient is highly unsatisfactory. Every pediatrician who is in a position to order drugs for icteric newborns and prematures would appreciate the possibility to calculate at least the total amount of benzoic acid derivatives which the child receives in one day.

We have not found in our experiments any indications for displacement of bilirubin by aminoglycosides. Possibly, the varying data in the literature may be explained in part by the various additives of stabilizers. However, we cannot prove this because not all publications list the specific commercial preparations under study.

A comparison of the change in serum bilirubin content after Refobacin® 10 mg (1973/74 production) and benzyl alcohol in 3–5 day old and 3–4 week old homozygote Gunn rats has biological and clinical interests in regard to the so-called maturation of metabolic functions and should cause a further standarization of the animal model used. Neither in newborn humans [28] nor in young rats [4] the degradation of benzoate to hippurate occurs with the same speed seen in older individuals. Also the hippuric acid excretion by the kidney is decreased in the neonate of both species [10, 14, 21].

DAVIS and YEARY [7] studied in two day old and in adult homozygous Gunn rats the fate of labelled bilirubin after displacement from the plasma-albumin bond by sulfadimethoxin. The percent distribution in the different tissues varied between young and old animals. Adult animals had a marked accumulation in the liver while in young animals it appeared in the gastro-intestinal tract (a probable explanation is the lack of Y and Z proteins in the liver of the young animals). In both adult and newborn rats the dye appeared in the brain. Only in young rats was there a marked difference in the LD_{50} of sulfadimethoxin between homozygous (icteric) and heterozygous (non-icteric) rats – 63 mg/kg versus 770 mg/kg. The authors concluded a higher sensivity for the toxic action of free bilirubin, in the young animals.

In testing drugs on Gunn rats one has to expect different results between adult and young animals of varying ages. It is not clear which age group: 2, 5, or even 10 days old rats represent best the conditions in newborn and premature infants (see 8).

Summary

The effect of several antibiotics of aminoglycoside structure on the albumin binding of bilirubin has been tested in homozygous (jaundiced) Gunn rats aged 3–5 days. The following drugs were investigated: different preparations of gentamycin, kanamycin, tobramycin and sisomicin. The animals received 50–75 % of the LD_{50} of heterozygous (non-jaundiced) Gunn rats. Mortality, weight gain and changes in the plasma bilirubin concentration were recorded.

It was found that the displacement of bilirubin from albumin is caused by the different stabilizers used and not by the antibiotic itself. With the exception of lyophilized preparations of gentamycin for intrathecal application all vials contain different amounts of these preservatives. Special preparations used during the newborn period contain relatively more of these stabilizers.

The toxicity of the additives has already a negative influence on the LD_{50} for heterozygous Gunn rats when

the low dosed Refobacin® and Sulmicin® vials are given. For Refobacin® (production 1973/74) the tolerance is reduced by nearly 50%. The toxicity caused by the stabilizer alone is even more marked when given to homozygous (jaundiced) Gunn rats. It becomes evident that benzylalcohol is the substance responsible for the displacement of bilirubin from albumin. The serum concentration of bilirubin decreases for 3–24 hrs depending on the doses given to the animal. This offers the opportunity to measure the competitive displacement of bilirubin easily and exactly.

The free, unbound, unconjugated bilirubin tends to diffuse into the lipid of the brain with resultant kernicterus. This was shown in histochemical preparations of the cerebellum of young homozygous Gunn rats. Using enzyme reactions for lactic acid dehydrogenase and $NADH_2$-tetrazolium reductase the cytotoxic effect of bilirubin on PURKINJE cells could be demonstrated.

The effect of the stabilizers used in the other antibiotic drugs tested can be neglected under clinical conditions.

Finally the steepness and duration of the decrease of plasma bilirubin after injection of the dangerous stabilizers was studied in animals of different age (1–5 days, 3–4 weeks). Different results observed can be explained by the more rapid metabolism of benzoates in older animals. However, it remains an open question at what age Gunn rats reflect most precisely the human situation in premature and newborn babies.

Keywords: Gunn rat, drugs (bilirubin-albumin binding), neonatal hyperbilirubinemia.

Zusammenfassung

Der Einfluß von verschiedenen Aminoglykosid-Präparaten auf die Bilirubin-Albumin-Bindung.

3–5 Tage alte homozygote (ikterische) Gunn-Ratten erhielten eine hochdosierte Injektion eines Aminoglykosid-Antibioticums aus verschiedenen Handelspräparaten. Getestet wurden: Gentamycin, Kanamycin, Tobramycin und Sisomicin. Die verabreichte Menge entsprach 50 bis 75% der LD_{50} für gleich alte heterozygote (nicht ikterische) Gunn-Ratten. Letalität, Gewichtsansatz und Veränderungen der Serumbilirubinkonzentration nach der Injektion wurden registriert.

Es konnte klar herausgestellt werden, daß eine Bilirubinverdrängung aus der Albuminbindung durch die antibiotischen Mittel selbst nicht eintrat.

Abgesehen von den für intrathekale Applikation vorgesehenen Ampullen, in denen das Antibioticum in lyophilisierter Form vorliegt, enthalten die Handelspräparate unterschiedliche Mengen von Zusatzstoffen (Stabilisatoren). Bemerkenswerterweise sind diese, auf den Wirkstoff berechnet, in den für Säuglinge bestimmten Ampullen, die relativ kleine Dosen des Antibioticums enthalten, in größerer Menge enthalten als in den Ampullen für Erwachsene.

Bereits bei heterozygoten Tieren wird die akute Toxicität – die LD_{50} – durch die Zusatzstoffe in den niedrig dosierten Refobacin®- und Sulmycin®-Ampullen beeinflußt. Beim Refobacin® 10 mg (Produktion 1973/74) wird die Verträglichkeit fast um die Hälfte reduziert. Noch markanter ist die durch den Stabilisator des Refobacin® 10 mg (Prod. 1973/74) bedingte Toxicität bei homozygoten Tieren. Es wird nachgewiesen, daß der darin enthaltene Benzylalkohol bei der ikterischen Ratte Bilirubin aus der Albuminbindung verdrängt. Die Serumbilirubinkonzentration sinkt parallel zur Dosis für mehr als 3 oder für mehr als 24 Stunden ab. Das Ausmaß der kompetitiven Bilirubinverdrängung kann auf diese Weise verhältnismäßig leicht und exakt erfaßt werden. Das freiwerdende Bilirubin verstärkt durch seine Neurotoxicität den Kernikterus. Das konnte eindeutig bei homozygoten jungen Ratten demonstriert werden, die mehrere niedriger dosierte Injektionen erhielten. Mit Hilfe histochemischer Reaktionen für Lactatdehydrogenase und $NADH_2$-Tetrazoliumreduktase konnte eine verstärkte Schädigung der PURKINJE-Zellen nachgewiesen werden.

Die Stabilisatoren aller übrigen getesteten Präparate können als relativ unbedenklich angesehen werden.

Schließlich wurden Steilheit und Dauer des Bilirubinabfalls nach Injektion der als kritisch bewerteten Stabilisatoren bei 3 bis 4 Wochen und 3 bis 5 Tage alten ikterischen Gunn-Ratten verglichen. Es zeigten sich Unterschiede, die auf den bekannten schnelleren Metabolismus von Benzoaten bei den älteren Tieren zurückzuführen sein dürften. Die Frage bleibt offen, in welchem Alter Gunn-Ratten die Verhältnisse bei neugeborenen bzw. frühgeborenen Menschen am ehesten wiederspiegeln.

Schlüsselwörter: Gunn-Ratte, Medikamente (Bilirubin-Albumin-Bindung), Neugeborenenhyperbilirubinämie.

Résumé

Influence de diverses préparations d'aminoglycoside sur la liaison bilirubine/albumine

Des rats «Gunn» agés de 3 à 5 jours, homozygotes (ictériques), ont reçu une injection fortement dosée de différentes préparations commerciales d'un antibiotique aminoglycoside afin de tester la Gentamycine, la Kanamycine, la Tobramycine et la Sisomicine. La dose appliquée correspondait à 50 ou 75% de la LD_{50} pour la même race de rats, âgés également de 3 à 5 jours, mais hétérozygotes (non ictériques)' On a enregistré après injection la létalité, la prise de poids et les changements des concentrations de la bilirubine du sérum.

On a pu prouver que les antibiotiques mêmes ne «déplaçaient» pas la bilirubine liée aux albumines du sérum.

En dehors des ampoules prévues pour l'application intrathécale et dans lesquelles l'antibiotique se trouve sous forme lyophilisée, les autres préparations commerciales contiennent des quantitées différentes d'additifs ou stabilisateurs. Il est intéressant de relever que, bien que renfermant des doses relativement faibles d'antibiotique, les ampoules prévues pour les nourrissons contiennent davantage de ces matières stabilisatrices que les ampoules réservées aux adultes.

Même chez les animaux hétérozygotes, la létalité (LD_{50}) est influencée par les stabilisateurs dans les ampoules à dose minime de Réfobacine® et Sulmycine®. La tolérance de la Réfobacine® à 10 mg (production 1973/74) est presque réduite de moitié. Chez les animaux homozygotes, la toxicité causée par le stabilisateur de la Réfobacine® à 10 mg (production 1973/74) est encore plus marquante. **Il a été établi que chez le rat ictérique, l'alcool benzylique, contenu dans ce stabilisateur, déplace la bilirubine fixée à l'albumine.** La concentration de la bilirubine du sérum baisse parallèlement à la dose injectée pour plus de 3 ou pour plus de 24 heures. De cette manière, on peut mesurer d'une façon relativement simple et précise le déplacement compétitif de la bilirubine. **La bilirubine non liée renforce par sa neurotoxicité l'ictère nucléaire,** ce qui a pu être démontré clairement chez des jeunes rats homozygotes ayant reçu plusieurs doses plus faibles. Diverses réactions histochimiques pour lactatdéhydrogénase et $NADH_2$-tetrazoliumréductase ont permis de démontrer une détérioration importante des cellules de PURKINJE du cervelet.

Les stabilisateurs de toutes les autres préparations sont relativement insignifiants.

Enfin on a comparé la durée et la vitesse de la baisse du taux de bilirubine après injection de certains stabilisateurs jugés critiques chez des rats Gunn ictériques agés de 3 à 4 semaines et de 3 à 5 jours. Les différences constatées semblent s'expliquér par le métabolisme accéléré des benzoates chez les animaux plus agés. On n'a toujours pas résolu la question quel est l'âge des rats Gunn qui reflète le mieux la situation chez les enfants nouveaux-nés ou prématurés.

Mots clés: Rats Gunn, médicaments (la liaison bilirubine/albumine), hyperbilirubinémie des nouveauxnés.

During the time of editing we tested one more aminoglycosid i. e. Amikacin (Biklin® Grünenthal/Bristol). Using the 100 mg vials (for babies) serumbilirubin did not decrease at all after an injection of 500 mg/kg. When 1000 mg/kg were given, a rather small decrease (about 10% of the starting level) could be noticed. The manufacturers informed us that neither the stabilizers of the formulation for babies nor for adults contain benzoates or benzylalcohol.

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